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In-Utero and Neonatal Exposure to Secondhand Smoke Causes Vascular Dysfunction in Newborn Rats

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Objectives. **We sought to determine the effects of secondhand smoke (SHS) exposure on vascular reactivity in newborn and infant rats.**

Background. **Secondhand smoke exposure increases cardiovascular risk. Secondhand smoke–induced endothelial dysfunction has been demonstrated in older teenagers and young adults. We have previously shown in adult rabbits that SHS induces atherogenesis and endothelial dysfunction. The effects of SHS on vascular function in the offspring of SHS-exposed mothers and in infants are unknown.**

Methods. **In this study the effects of in-utero (21 days) and neonatal (28 days) exposure to SHS were examined in 80 rats, 4 weeks of age, in a 2-by-2 design study. Rats were exposed to sidestream smoke in smoking chambers. Aortic rings were excised and isometric force responses to phenylephrine, acetylcholine, A23187 and nitroglycerin were studied in organ baths.**

Results. **Neonatal SHS exposure reduced animal weight ($p = 0.009$). In-utero exposure increased the sensitivity (decreased the EC50) of aortic rings to phenylephrine ($p < 0.0005$), as did neonatal exposure ($p = 0.01$). Maximal contraction to phenylephrine was reduced by in-utero exposure ($p = 0.04$). In-utero SHS exposure reduced maximal endothelium-dependent relaxation to acetylcholine ($p = 0.04$) and increased the EC50 ($p = 0.05$), suggesting impaired sensitivity to acetylcholine. In-utero exposure decreased the sensitivity (increased the EC50) to the endothelium-independent vasodilator nitroglycerin ($p = 0.003$).**

Conclusions. **Secondhand smoke has detrimental effects on vascular smooth muscle function in the newborn.**

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Secondhand tobacco smoke (SHS) is the third largest preventable public health risk after active smoking and alcohol (1). Epidemiologic studies demonstrate an increase in coronary artery disease events and mortality with exposure to SHS (2). There is increasing concern about the potential impact of tobacco-related disease before adulthood. Secondhand smoke exposure in childhood reduces high-density lipoprotein levels (3). In teenagers and young adults SHS exposure impairs endothelium-dependent relaxation (4). The American Heart Association has formally concluded that SHS constitutes an important health risk for both adults and children (5). It is not known whether exposure to SHS even earlier in life, such as in utero or in infancy, or both, causes vascular dysfunction.

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Tobacco smoke metabolites such as cotinine can be recovered in the meconium and hair of neonates whose mothers had been exposed to SHS (6,7). Nursing mothers exposed to SHS also transfer cotinine and possibly other toxins from tobacco smoke to infants through breast milk (8,9). This study sought to determine the effect of SHS on the vasculature of infant rats whose mothers had been exposed to SHS during pregnancy, on the vasculature of rats exposed as neonates and the effects of SHS on neonatal weight.

Methods

Protocol. The study protocol was approved by the Committee for Animal Research of the University of California at San Francisco and was performed in accordance with the recommendations of the American Association for Accreditation of Laboratory Animal Care. Pregnant rats were housed in separate cages, and after delivery mother rats were also housed in separate cages with their pups. Baby rats were randomly assigned to receive, or not to receive, SHS exposure in four groups: 1) those whose mothers were exposed to SHS while pregnant (21 days) (SHS_U); 2) those whose mothers were exposed to SHS while pregnant, and who were then exposed to

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Abbreviations and Acronyms

EC50 = concentration for half-maximal contraction
Phe = phenylephrine
SHS = secondhand smoke

SHS 4 weeks after birth (SHS_{UN}); 3) those whose mothers had not been exposed to SHS during gestation (21 days), but who were then exposed for the first 4 weeks of life (SHS_N); and 4) those whose mothers had not been exposed to SHS and who were not exposed during the first 4 weeks of life (controls). Each mother gave birth to eight or nine pups.

As we have previously described, rats were exposed to cigarette sidestream smoke using a smoking machine in smoking chambers (10). Rats were housed in individual cages. Rats randomized to SHS were placed in SHS exposure chambers (BioClean, Duo. Flo, model H 5500; Lab Products Inc.) that measured $1.92 \times 1.92 \times 0.97$ m (3.58 m^3) and accommodated eight rats at a time. Rats were exposed to sidestream smoke from Marlboro filter cigarettes (four cigarettes every 15 min for 6 h/day, 5 days/week) using a smoking machine (Heinr, Borgwald GMBH RM 1/G, Hamburg, Germany) for 10 weeks, from week 3 to week 13. Three fans in the exposure chambers were adjusted to ensure good mixing of the air within them. At the end of the 6-h exposure period the exhaust fan on the BioClean unit was turned on. This rapidly lowered the level of SHS pollution in the exposure chamber to background levels corresponding to those of the non–SHS-exposed animals until the next day when the BioClean unit was turned off and the smoking machine was turned on again. Rats randomized to nonexposure were housed in separate cages in the same type of exposure chamber in another room, but without a smoking machine.

At week 4 after birth rats were sacrificed by lethal injection with intravenous pentobarbital, 130 mg/kg body weight. The age of 4 weeks was chosen because rats of this age weighed approximately 100 g and had aortas of sufficient size to be easily studied in organ baths. Aortas from the largest 20 pups from each group were studied (n = 20 all groups). One arterial ring segment (1 to 2 mm in diameter and 5 mm in length) was rapidly excised starting from the descending thoracic aorta. Rings were taken from the same position in the aorta for each study.

Vascular reactivity studies. Each ring was suspended horizontally between two parallel stainless steel wires to measure isometric force in individual organ baths containing Krebs solution at 37°C. Isometric force generated by the ring segment was recorded continuously as previously described (10).

Ring segments were stabilized at 1 g rest force for 60 min before being studied. This rest force was determined in a prior series of organ bath experiments which determined that this rest tension allowed for the greatest tension changes with vasoactive agents. To measure responsiveness to phenylephrine (Phe) and to calculate the dose needed for precontraction for relaxation dose–response curves for each individual ring, Phe in increasing doses (from 10^{-9} to 10^{-4} mol/L) was added to each ring/bath. For each ring the dose needed to achieve half-maximal contraction ($EC50_{Phe}$) was calculated. After the Phe contraction series the baths were washed out three times with fresh Krebs solution and the rings were allowed to stabilize for 1 h. To study endothelium-derived nitric oxide–mediated vasorelaxation, aortic rings that had been precontracted with the $EC50_{Phe}$ for that ring were exposed to the endothelium-dependent vasodilator acetylcholine (doses from 10^{-9} to $10^{-4.5}$ mol/L) and later to the muscarinic receptor-independent endothelium-dependent vasodilator calcium ionophore A23187. Lastly, each ring was precontracted and exposed to the endothelium-independent relaxation agent nitroglycerin (doses from 10^{-9} to 10^{-5} mol/L). At the end of each series the baths were washed out twice with fresh Krebs solution and the rings were allowed to stabilize at baseline force. Vascular reactivity experiments were performed by an investigator who was unaware of the treatment group.

Drugs and their sources. Phenylephrine, acetylcholine and the calcium ionophore A23187 were purchased from Sigma Corp. (St. Louis, Missouri). Nitroglycerin was purchased from Solopak Laboratories Inc. (Elk Grove, Illinois). Distilled water was used as the solvent for all agents other than A23187, which was dissolved in dimethyl sulfoxide to create a stock solution of A23187, which was then sequentially diluted with distilled water.

Serum nicotine and cotinine. Serum nicotine and cotinine levels were measured in an additional 26 rats at 4 weeks after birth (six or seven rats per group) using nitrogen–phosphorus gas chromatography.

Statistical analysis. All results are expressed as mean ± SEM unless otherwise indicated. The response to Phe was expressed as change in force from baseline (grams) and recorded and analyzed as described previously. Relaxation of aortic rings is expressed as percentage change of net developed force (measured force − baseline force)/(precontracted force − baseline force), EC50 and slope. Slope, expressed by the Hill coefficient, was calculated using commercially available software (Kaleidagraph; Abelbeck Software) that resolved a curve of best fit to each ring's dose–response relationship. Slope was calculated to describe sensitivity to vasoactive substances. The effects of in-utero and neonatal SHS exposure on vascular reactivity and animal weight were evaluated using a two-way analysis of variance, including main effects in-utero exposure (present or absent) and neonatal exposure (present or absent), using a general linear model and commercially available software (Minitab 10.51; Minitab Inc., State College, Pennsylvania). Differences in birth mortality were assessed using the chi-square test. A p value of less than 0.05 was considered significant.

Results

Birth mortality was greater in rats exposed in utero than in rats not exposed in utero (12% vs. 3%; $p < 0.001$). Neonatal

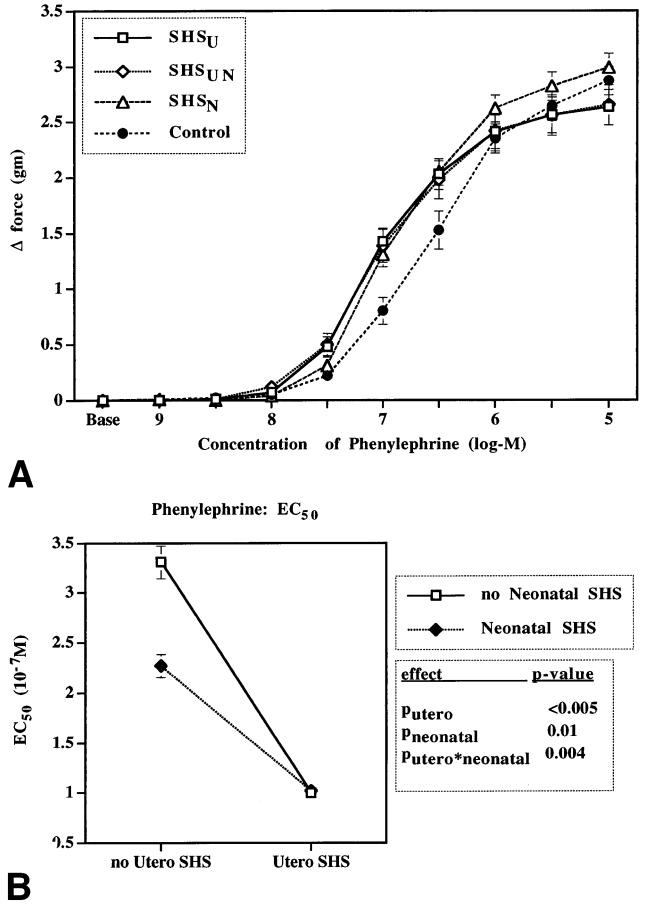

Figure 1. (**A**) Dose–response curves of rat aortas exposed to Phe. The dose–response curves of the three groups of SHS-exposed rats are all shifted to the left of that of the control group, indicating greater sensitivity to adrenoceptor-mediated contraction. (**B**) EC50 values for Phe in the four groups of rats. Both in-utero and neonatal tobacco smoke exposure reduce the EC50 to Phe. SHS_U = in-utero secondhand smoke exposure; SHS_N = neonatal secondhand smoke exposure; SHS_{UN} = in-utero and neonatal secondhand smoke exposure; Control = no secondhand smoke exposure.

SHS exposure was associated with reduced average body weight (104 ± 0.4 vs. 111 ± 0.4 g; p = 0.009) at 4 weeks. In-utero exposure did not influence body weight when measured at 4 weeks (107 ± 0.4 vs. 108 ± 0.4 g; p = 0.97). Neonatal SHS-exposed rats had significantly greater plasma cotinine (437 ± 124 ng/ml vs. undetectable; i.e., <0.1 ng/ml; p < 0.0001) and nicotine (39 ± 5 ng/ml vs. undetectable; p < 0.0001) levels, confirming the presence of tobacco-related compounds in the circulation of the newborn rats.

Mean dose–response curves to Phe are plotted in Figure 1A. In-utero and neonatal exposures were both associated with increased sensitivity (lesser EC50) to Phe (Fig. 1B, Table 1). In-utero exposure reduced maximal contraction to Phe (p = 0.04). There was a significant interaction between in-utero and neonatal smoke exposure on the EC50 of Phe, indicating that the effect of in-utero SHS exposure on sensitivity of smooth muscle contraction was dominant over the effect of neonatal SHS. In-utero SHS exposure reduced maximal acetylcholine-induced endothelium-dependent relaxation (Fig. 2A, Table 2). In-utero exposure tended to increase the EC50 of the dose–response curves to A23187 (Fig. 2B), suggesting reduced sensitivity to this endothelium-dependent vasorelaxing agent. In-utero SHS exposure also caused a rightward shift of the dose–response curves to nitroglycerin, indicating reduced smooth muscle sensitivity in response to an endothelium-independent relaxing agent (Fig. 3A and B).

Other than the interaction term described above, none of the interaction terms of the general linear model analysis of variance achieved significance, indicating that the effects of in-utero exposure did not modify the effects of neonatal exposure, or vice versa.

Discussion

This is the first study to present evidence that maternal SHS exposure during pregnancy and neonatal SHS exposure cause abnormal vasoconstrictor and vasodilator responses in infant rats. The 21-day gestational period of rats was sufficient for these vascular abnormalities to develop. We have previously shown in adult rabbits that 10 weeks of exposure to SHS at the same dose caused abnormalities of endothelial function (10), and that 6 weeks of SHS exposure of adult rats increased myocardial infarct size (11). The shorter period in which vascular abnormalities were seen in baby rats suggests that the developing cardiovascular system may be even more sensitive to the injurious influences of tobacco smoke exposure. The impairment of endothelium-dependent vasorelaxation in the context of concurrent abnormalities of endothelium-independent relaxation suggests that smooth muscle dysfunc-

Table 1. Vasocontraction

	Group				p Value		
	SHS_U	SHS_{UN}	SHS_N	Control	Utero	Natal	Utero-Natal
n	20	20	20	20			
Maximal Δ force (g)	2.64 ± 0.16	2.68 ± 0.18	3.03 ± 0.13	2.92 ± 0.14	0.038	0.659	0.85
Slope	1.3 ± 0.03	1.2 ± 0.02	1.1 ± 0.03	1.1 ± 0.04	0.769	0.820	0.27

The p values presented are the principal effects ("utero" and "natal") as determined by the general linear model analysis of variance and the interaction term ("utero × natal"). SHS_U = in-utero secondhand smoke exposure; SHS_N = neonatal secondhand smoke exposure; SHS_{UN} = in-utero and neonatal secondhand exposure; Control = no secondhand smoke exposure.

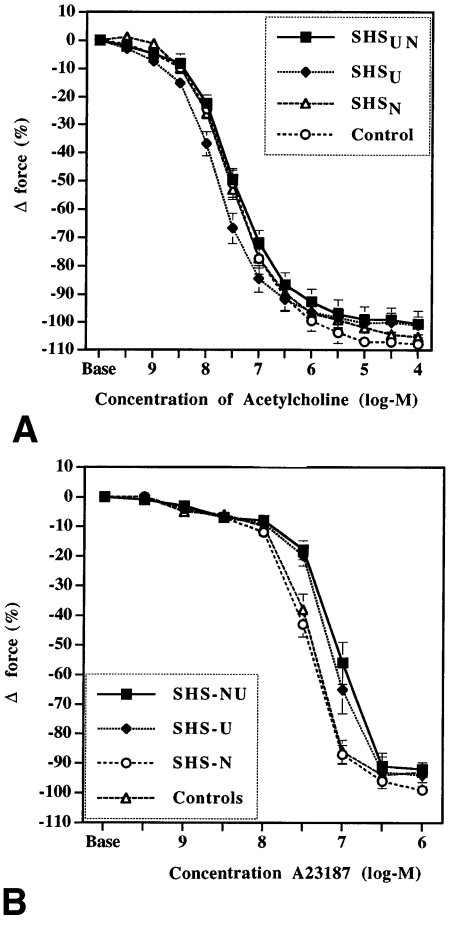

Figure 2. Endothelium-dependent relaxation. **(A)** Dose–response curves of rat aortas exposed to increasing concentration of acetylcholine. In-utero SHS exposure reduced maximal relaxation. **(B)** Dose–response curves of rat aortas exposed to increasing concentration of the calcium ionophore A23187. In-utero SHS tended to shift the EC50 of the response to the right, indicating reduced sensitivity.

tion underlies the observed abnormalities. Enhanced vasoconstrictor sensitivity in response to Phe was observed in this study, consistent with published studies of enhanced vasoconstriction in active smokers (12).

The mechanisms by which maternal and neonatal exposure to SHS cause the observed vascular abnormalities are undefined. Maternal exposure to SHS results in absorption of toxins, which are transmitted to the fetus from the mother. Hair accumulation of cigarette components is reflective of long-term exposure to tobacco smoke (6). There is a proven correlation of maternal and neonatal infant hair levels of nicotine ($p < 0.001$) and cotinine ($p < 0.0001$) (6). There is increased urinary excretion of cotinine by infants who are breast-fed by mothers exposed to SHS (8); thus infants may be exposed to SHS toxins by inhalation and also through their diet if they are breast-fed by a mother exposed to SHS. Maternal SHS exposure results in similar elevations in meconium nicotine metabolite concentrations, as does mild to moderate maternal active smoking (7), and may therefore be viewed as a similar toxicologic phenomenon. A recent study by Galanti et al. (13) suggested that the highest cotinine levels among those exposed to SHS occur in newborns rather than in exposed adults and children. Nicotine, cotinine or other vasoactive substances in tobacco smoke that are transferred to the fetus and to the nursing infant may adversely influence developing vascular smooth muscle.

The increasing incidence of tobacco smoking by adolescent females has engendered rising concern of the long-term health consequences of tobacco smoke exposure earlier in life. Because risk of tobacco smoke–induced vascular disease is related to total lifetime smoke exposure, beginning smoking earlier in life may increase total lifetime smoke exposure and therefore risk of vascular disease. Although the data presented in this study were obtained in an animal model, they do

Table 2. Vasorelaxation

	Group				p-Value		
	SHS$_U$	SHS$_{UN}$	SHS$_N$	Control	Utero	Natal	Utero × Natal
n	20	20	20	20			
ACh							
Max	−102.3 ± 3.8	−98.6 ± 3.6	−105.8 ± 2.4	−108 ± 3.5	0.04	0.33	0.85
EC50	$6.1 \times 10^{-8} \pm 3.5 \times 10^{-8}$	$4.6 \times 10^{-8} \pm 9.6 \times 10^{-9}$	$4.6 \times 10^{-8} \pm 9.6 \times 10^{-9}$	$4.0 \times 10^{-8} \pm 1.2 \times 10^{-8}$	0.80	0.57	0.22
Slope	1.10 ± 0.12	0.97 ± 0.11	1.13 ± 0.09	0.97 ± 0.08	0.75	0.45	0.53
A23187							
Max	−98 ± 4	−98 ± 3	−96 ± 3	−95 ± 3	0.534	0.08	0.69
EC50	$1.5 \times 10^{-7} \pm 2.7 \times 10^{-8}$	$1.6 \times 10^{-7} \pm 1.1 \times 10^{-8}$	$3.8 \times 10^{-8} \pm 3.0 \times 10^{-9}$	$3.3 \times 10^{-8} \pm 2.2 \times 10^{-8}$	0.05	0.37	0.18
Slope	2.61 ± 0.29	3.4 ± 0.60	2.8 ± 0.60	5.17 ± 1.55	0.13	0.11	0.07
NTG							
Max	−98 ± 3	−98 ± 4	−96 ± 3	−98 ± 3	0.57	0.85	0.88
Slope	0.84 ± 0.11	2.61 ± 0.30	0.94 ± 0.17	1.2 ± 0.06	0.36	0.08	0.45

The p values presented are the principal effects ("utero" and "natal") as determined by the general linear model analysis of variance and the interaction term ("utero × natal"). A23187 = calcium inophore–induced endothelium-dependent relaxation; ACh = acetylcholine-induced endothelium-dependent relaxation; max = maximal change in force; NTG = nitroglycerin-induced endothelium-independent relaxation other abbreviations as in Table 1.

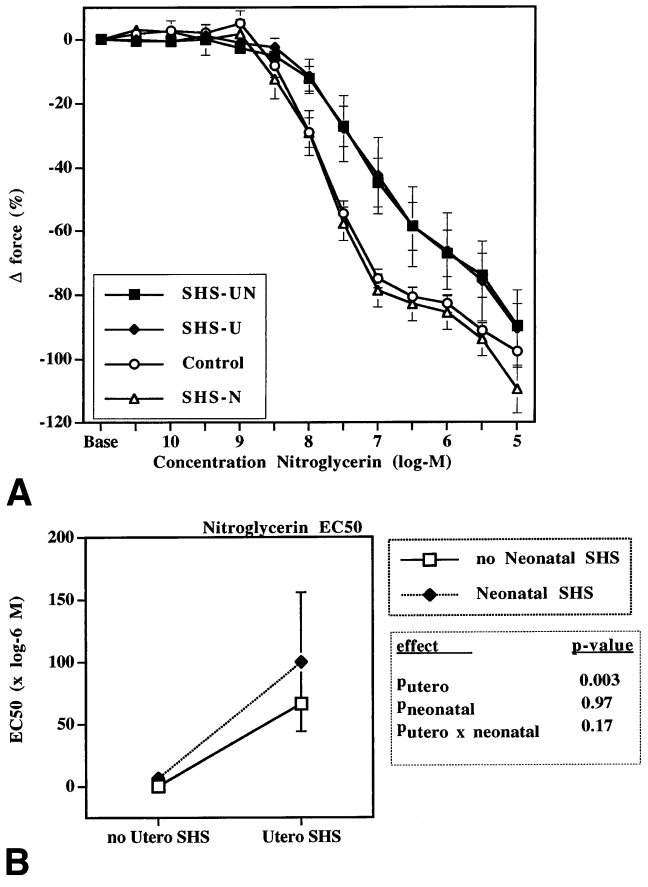

Figure 3. Endothelium-independent relaxation. **(A)** Dose–response curves of rat aortas exposed to nitroglycerin. The dose–response curves of the in-utero SHS exposed rats are all shifted to the right of the control and neonatal SHS groups. **(B)** Values of EC50 for nitroglycerin in the four groups of rats. In-utero tobacco smoke exposure increased the EC50 to nitroglycerin, indicating less sensitivity to endothelium-independent relaxation. Abbreviations as in Figure 1.

indicate that tobacco smoke induces abnormal vascular responses very early in life. The other major concern engendered by the increasing incidence of tobacco smoking by adolescent females pertains to issues of infant health. In this study we observed greater birth mortality among rats exposed to SHS in utero and lower body weight in infant rats exposed to SHS. Previous human studies have demonstrated that maternal tobacco smoke exposure is injurious to fetuses in utero and to neonates. Maternal smoke exposure, active or passive, is associated with preterm labor (14), threatened abortion (14), increased risk of spontaneous abortion (15) and low birth weight (16). Secondhand smoke exposure in infants is associated with sudden infant death syndrome (9).

Study limitations. The data presented in this study are descriptive. No mechanism for the abnormal vascular responses was determined. The tobacco smoke exposures (air nicotine and carbon monoxide) in our model are about twofold higher than in heavy human smoking environments (17). An approximate equivalent to the volume of our smoking chamber (3.6 m^2) would be the interior volume of an average automobile sedan (3.7 m^2). However, the measurements of plasma nicotine and cotinine in this study were similar to those of average smokers (15 to 40 cigarettes/day) (18,19). As our study was limited to the largest 20 animals of each group, it may have included bias.

Conclusions. Neonatal SHS exposure retards growth and results in an increased sensitivity to vasoconstrictive stimuli. Secondhand smoke exposure to the fetus also causes an increased sensitivity to vasoconstrictor stimuli and impaired vasorelaxation. The fetus is not protected in utero from the effects of SHS, and in fact may be uniquely susceptible to SHS-induced vascular smooth muscle dysfunction.

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